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PREVALENCE OF INFECTION WITH *FRANCISELLA TULARENSIS*, *BORRELIA BURGDORFERI* SENSU LATO AND *ANAPLASMA PHAGOCYTOPHILUM* IN RODENTS FROM AN ENDEMIC FOCUS OF TULAREMIA IN BULGARIA

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Abstract: The prevalence of *Francisella tularensis*, *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in rodents is a determinant for their role in maintaining pathogens in the environment. A total of 169 rodents, trapped in an endemic focus of tularemia, were examined by PCR to asses the frequency of infection with the etiological agents of tularemia, Lyme borreliosis and human granulocytic anaplasmosis. The overall prevalence of *F. tularensis* was 22%. In 6% of the black rats, *F. tularensis* was identified in coinfection with *B. burgdorferi* and in 3% in coinfection with *A. phagocytophilum*. *B. burgdorferi* and *A. phagocytophilum* were detected in 26% and 8% of the trapped rodents. Results obtained show the existence of an active natural focus of tularemia. The high level of coinfections indicated that the endemic focus is mixed and generates risk for multiple infections in humans. Further investigations are needed to reveal interactions between the pathogens in the infected animals.

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INTRODUCTION

Many mammal species have now been determined to maintain *Francisella tularensis* in the environment [2, 13, 21]. It has been noted that epizootics in wild animals precede outbreak of tularemia in humans [6, 15, 17]. As an illustration, the outbreak of tularemia in Bulgaria during 1998-2003 was associated with increased rodents population. Tularemia occurs in endemic foci, where three genera of ticks - *Dermacentor*, *Ixodes* and *Haemaphysalis* transmit *Francisella tularensis* between mammalian hosts [4, 5].

Various rodent species have been implicated as natural reservoirs for *Borrelia burgdorferi* sensu lato [1, 11, 12]. Lyme borreliosis distribution follows occurrence of the vector Ixodes ticks and can be found throughout the Northern Hemisphere [2, 13, 15].

The tick vectors of *Anaplasma phagocytophilum*, the etiological agent of human granulocytic anaplasmosis (HGA), are also *Ixodes* ticks and coinfections in patients have been documented [17, 21]. Small mammals, being common hosts of *Ixodes* ticks, are suspected of being natural reservoirs for *A. phagocytophilum*. However, reservoirs of HGA have not been determined. In the USA, white-footed mice, *Peromyscus leucopus*, are implicated as a reservoir for A. *phagocytophilum* [3, 10, 22, 26, 27]. In Europe, very little is known about animal reservoirs of HGA. *A. phagocytophilum* was found in tissues and blood

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from bank vole (*Clethrionomys glareolus*), and in tissues from the wood mouse (*Apodemus sylvaticus*), yellow-necked mouse (*Apodemus flavicollis*), and common shrew (*Sorex araneus*) [9, 14].

An endemic focus of tularemia has appeared in Bulgaria since 1998 in the vicinity of Slivnitza region, west Bulgaria. A total of 262 tularemia cases were reported between 1998–2003. As a part of the investigations into the endemic focus, rodents were captured and their tissues tested by PCR to detect infection with *F. tularensis* and tick-borne pathogens *B. burgdorferi* and *A. phagocytophilum*. The goal of our study was to show the epidemiological role of rodents, as well as to reveal possible coinfections and asses the risk for public health.

MATERIALS AND METHODS

Samples collection. Rodents were trapped in the endemic for tularemia area near the western border of Bulgaria during September–December 2002. This region is located in a valley, surrounded by mountains. Animals were collected by live traps, placed in homes, outbuildings, gardens, and fields. Captured rodents were identified to species level and euthanized. Spleen samples were collected and stored at -20ºC until use.

DNA purification. DNA was extracted from animal tissue with GenomicPrep Cells and Tissue DNA Isolation Kits (Amersham Bioscience, UK). The protocol used was that suggested by the manufacturer. Extracted DNA was eluted in 100 µl of hydration solution.

PCR amplification. For detection of *F. tularensis*, reaction mixture used 5 µl DNA extraction sample as the template in a total volume of 25 µl. The reaction mixture (puRe Taq Ready-To-Go PCR Beads, Amersham Bioscience) contained 2.5 units of pure Taq DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl2, 200µM dATP, dCTP, dGTP, and dTTP. The primer pair was Tul4-435 and Tul4-863, amplifying a 0.4 kb fragment of the 17 kDa lipoprotein gene of *F. tularensis*. Cycling conditions were as described by Sjöstedt [18]. Amplified products were analyzed by agarose gel electrophoresis in 1.5% agarose gel stained with ethidium bromide.

For detection of *A. phagocytophilum*, a similar reaction mixture was used with primers LA1 and LA6, amplifying the *epank1* gene of *A. phagocytophilum* under conditions as described by Walls *et al.* [24]. Amplified products were visualized in 1.5% agarose gel electrophoresis.

For detection of *B. burgdorferi*, ProDect *B. burgdorferi* kit was used according to the instructions of the manufacturer. 10 µl DNA samples were added to 90 µl amplification mixture containing (at a final concentration) 2.5 units DNA polymerase, 50 µl (each) primers, 0.2 mM (each) dNTPs, 1.75 mM $MgCl_2$ (Bioanalisi CentroSud, Italy). Primers Fla1 and Fla3 amplified the flagelin gene. Cycling conditions involved an initial 4 min. denaturation at 94ºC, followed by 45 cycles, each consisting of denaturation for 45 sec. at 94ºC, primer annealing to the template at 55ºC for 45 sec., and primer extension at 72ºC for 45 sec., and final extension for 5 min. at 72ºC. Amplified DNA was detected by DNA enzyme immunoassay kit (GEN.E.I.A.®, Bioanalisi Centro Sud, Italy), based on the hybridization of amplified DNA with a single-stranded DNA probe, coated on the wall of the microtitre plate wells and detection of the hybrid with anti-double-stranded DNA mouse monoclonal antibody.

RESULTS

A total of 169 small rodents were trapped in an endemic focus of tularemia and spleen samples were examined for the presence of *F. tularensis*, *B. burgdorferi* sensu lato and A. *phagocytophilum* (Tab. 1).

F. tularensis DNA was detected in 32 (23.5%) of the 136 captured black rats (*R. rattus*). In 20 of these 32 rodents, *F. tularensis* was detected as a sole pathogen, and in 12 *F. tularensis* was identified in coinfection with *B. burgdorferi* (n=8) and *A. phagocytophilum* (n=4). In addition, *F. tularensis* DNA was also detected in 5

Table 1. PCR detection of *F. tularensis*, *B. burgdorferi* sensu lato and *A. phagocytophilum* DNA in rodents, captured in an endemic focus of tularemia.

Pathogens detected	Animal species collected No. of positive (% positive)			Total positive samples	
	Rattus rattus (n=136)	*Mus musculus* (n=24)	*Apodemus agrarius* (n=9)	No.	%
Only *F. tularensis*	20(14.7)	5(20.8)	0	25	14.8%
Only *B. burgdorferi*	32(23.5)	4(16.7)	0	36	21.3%
Only *A. phagocytophilum*	6(4.4)	0	3(33.3)	9	5.3%
F. tularensis + *B. burgdorferi*	8(5.9)	0	0	8	4.7%
F. tularensis + *A. phagocytophilum*	4(2.9)	0	0	4	2.4%
Total *F. tularensis*	32(23.5)	5(20.8)	0	37	21.9%
Total *B. burgdorferi*	40(29.4)	4(16.7)	0	44	26%
Total *A. phagocytophilum*	10(7.4)	0	3(33.3)	13	7.7%

(20.8%) of the 24 mice (*M. musculus*). In all 5 mice *F. tularensis* was found as the sole pathogen. No infection with *F. tularensis* was detected in black striped mice (*A. agrarius*). The overall prevalence of *F. tularensis* infection in trapped rodents was 21.9% (37/169).

Prevalence of *B. burgdorferi* infection was even higher. PCR assays of 40 *R. rattus* (29.4%) yielded evidence of *B. burgdorferi* sensu lato DNA, in 32 of the samples as the sole pathogen. Four (16.7%) of the 24 *M. musculus* were infected with *B. burgdorferi*. In all 4 mice, *B. burgdorferi* was the sole pathogen detected. The overall prevalence of *B. burgdorferi* infection in the rodents examined was 26% (44/169).

The lowest was the level of infection with *A. phagocytophilum* in the trapped animals (7.7%). *A. phagocytophilum* DNA was detected in 10 *R. rattus* (7.4%). In four of these cases, *A. phagocytophilum* was in coinfection with *F. tularensis*. No infection with this pathogen was found in *M. musculus*. However, this was the only pathogen found in *A. agrarius* mice. *A. phagocytophilum* DNA was detected in 3 (33.3%) of the 9 *A. agrarius*.

DISCUSSION

This report showed the role of three rodent species, *R. rattus*, *M. musculus* and *A. agrarius* in maintaining the agents of tularemia, Lyme borreliosis and human granulocytic anaplasmosis (HGA) in the environment. The results of this study confirmed the existence of mixed natural foci.

Our results suggested that *A. agrarius* was the preferable host for *A. phagocytophilum*. *R. rattus* and *M. musculus* were preferable hosts for *B. burgdorferi* and *F. tularensis* and infection with *A. phagocytophilum* was very unusual or not detected.

A number of papers have shown *Peromyscus* mice as the main reservoir host for *A. phagocytophilum* in the USA [10, 22, 25]. The equivalent role of *Apodemus* mice in Europe has been suspected [9, 14]. Our finding that one-third of *A. agrarius* mice were naturally infected with the agent of HGA suggested that this rodent species most probably serve as a natural reservoir of the pathogen.

Many mammal species have now been determined as competent reservoirs of *B. burgdorferi* in Europe. The most important probably are mice (*Apodemus* spp.) and voles (*Clethrionomys* spp.) [1, 7, 8, 12, 16, 19, 20, 28]. We have revealed the role of black rats (*R. rattus*) and thus confirmed previous findings [8, 11]. In addition, we have shown the role of mice M. *musculus* as a reservoir for the agents of Lyme borreliosis and tularemia by demonstration of 16.7% and 20.8% prevalence of the agents, respectively. The high level of infections in urban rodents creates a significant risk for public health. The tularemia outbreak in Bulgaria during 1998-2003 illustrates this observation. Most of the patients presented with oropharyngeal manifestation of tularemia, which indicated that the route of infection was consumption of contaminated food or water. Obviously, the increase in rodent population, seen in the last years, favoured environmental contamination with *F. tularensis*. Incidence of the infection with the agent of tularemia in the small mammals trapped in the endemic focus was much higher (up to 23.5%) (Tab. 1) than reported for an endemic region in Austrian and Slovakian borderland (up to 7.7%) [23].

Coinfections of *F. tularensis* with *B. burgdorferi* or *A. phagocytophilum* found in this study, demonstrated that the natural focus of tularemia is mixed and generates risk for multiple infections in humans. The level of coinfection in rodents with *F. tularensis* and *B. burgdorferi* was higher than reported in the study of Vyrostekova *et al.* (up to 5.9% *vs.* 2.1%) [23], emphasizing the activity of the focus. In addition, we also found coinfections of *F. tularensis* with *A. phagocytophilum* in 2.9% of the black rats. In contrast with that study, showing infection with *B. burgdorferi* in the examined rodents from May–August, and infection with *F. tularensis* from August–December, we detected a higher incidence of infection with *B. burgdorferi* than with *F. tularensis*, even in the late autumn. Hence, our findings could hardly support the hypothesis that epizootics of tularemia lowered the risk of borrelial infection [23]. The reasons for such a discrepancy could be in the different animal species examined. Interference between the agents of tularemia, Lyme borreliosis and HGA in animal host reservoirs are not elucidated. Further investigations are needed to detect and confirm positive or negative interactions of the pathogens and to establish whether this interference is associated with the animal species.

In conclusion, the prevalence of infection with *Francisella tularensis*, *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in urban rodents elucidated their role in maintaining pathogens in the environment. The high incidence of the infection with the agent of tularemia in the small mammals, trapped in the endemic focus, demonstrated rodents as important sources of human infection via food and water contamination. The high level of coinfections indicated that the natural focus of tularemia generates risk for multiple infections in humans.

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